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PINGUISONE DERIVATIVES FROM AN AXENIC CULTURE OF THE LIVERWORT *ANEURA PINGUIS*

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Key Word Index—*Aneura pinguis*; Metzgeriales; pinguisone; sesquiterpene; axenic culture; liverwort.

Abstract—Three new pinguisane-type sesquiterpenes, 6α-hydroxy-3-oxo-pinguis-5(10)-ene-11,6-olide, 6α-methoxy-3-oxo-pinguis-5(10)-ene-11,6-olide and 3-oxo-pinguis-5(10),6-diene-11,6-olide, together with pinguisone, have been isolated from axenic culture of the liverwort, *Aneura pinguis*. Their structures and relative stereochemistry were elucidated on the basis of spectroscopic evidence and chemical transformation.

INTRODUCTION

Liverworts are known to be a rich source of sesquiterpenes and diterpenes, including new structural types with various biological activities [1–4]. For example, pinguisone (**1**) and its biosynthetically related compounds have been isolated from many liverworts of the Metzgeriales including *Aneura pinguis* [5], and the Jungermanniales including *Porella platyphylla* [6], *Trichocoleopsis sacculata* [7, 8], *Ptychanthus striatus* [9], *Frullanoides densifolia* [10], *Trocholejeunea sandvicensis* [10], *Bryopteris filicina* [11], *Plagiochila ovalifolia* [12], *P. retrospectans* [12] and *Dicranolejeunea yoshinagara* [13]. Compound **1** is biosynthetically interesting since its biogenesis is difficult to explain simply by the isoprene rule. From this point of view, we have previously reported the biosynthesis of **1** in axenic cultures of gametophytes of *A. pinguis* by feeding of [2-^{13}C]acetate [14]. The labelling pattern indicated two-methyl migrations and C–C bond cleavage of the main chain in farnesyl diphosphate in the formation of **1**. In subsequent biosynthetic studies we found three new pinguisane type sesquiterpenes from an axenic culture of the gametophytes of *A. pinguis*, and this report deals with their isolation and structural elucidation.

RESULTS AND DISCUSSION

The origin and the axenic culture of *A. pinguis* have been previously reported [14]. The diethyl ether extracts of the axenic cultures of *A. pinguis* were separated into five fractions by column chromatography on Sephadex LH-20. Fraction 4 was further separated by a combination of vacuum liquid column chromatography and HPLC to afford compounds **2** (16.9 mg), **3** (4.6 mg) and **4** (5.6 mg), together with **1**.

Compound **2** consisted of colourless crystals (mp 84–85°) with a molecular formula $C_{15}H_{20}O_4$ from the EI-HR mass spectrum (*m/z* 264.1362, $[M]^+$, calc. 264.1362). The UV spectrum showed an absorption at 235 nm (log ε 4.48) for an α,β-conjugated γ-lactone (δ_C 171.2). The ^{1}H NMR spectrum displayed two singlet methyls (δ_H 0.72 and 0.75), two doublet methyls (δ_H 1.06 and 1.08, $J = 6.7$ Hz each) and a vinylic proton (δ_H 5.74 *d*, $J = 1.8$ Hz), together with six other protons. A broad D_2O exchangeable singlet at δ_H 3.75 was assigned to a hydroxyl group (3350 cm^{-1}). The ^{13}C NMR spectrum showed the signals of four methyls, two methylenes, three methines and six quaternary carbons, indicating one double bond (δ_C 171.8 and 115.7), one conjugated carbonyl group (δ_C 171.2) and one hemiacetal carbon (δ_C 105.0). The complete structure of **2** was achieved by following through ^{1}H and ^{13}C NMR assignment with ^{1}H–^{1}H COSY, ^{1}H–^{13}C COSY, ^{1}H–^{1}H homodecoupling, DEPT, NOESY and COLOC (Tables 1 and 2). The α,β-conjugated lactone ring of **2** was confirmed by H–H coupling (H-10 with H-4) observed by ^{1}H–^{1}H COSY and ^{1}H–^{13}C long-range couplings (H-10 to C-6, C-11, and C-15; H-15 to C-5) by COLOC. A $-CH_2CH(Me)-$ unit, of which the proton-line assignment was confirmed by ^{1}H–^{1}H COSY and ^{1}H–^{13}C COSY, belonged to a cyclopentane ring. Segments of $-CH_2-$ and $-CH(Me)-$ could be attributed to the cyclohexane ring. These segments were both confirmed by ^{1}H–^{1}H COSY, ^{1}H–^{13}C COSY and COLOC. Remaining connectivities among all segments were revealed by the long-range couplings

Table 1. ^{1}H NMR data for pinguisone derivatives (**2**–**4**)*

H	2	3	4
1	3.30 *m*	3.13 *m*	2.61 *m*
2	2α 2.65 *dd*, *J* = 9.8, 19.8	2α 2.64 *dd*, *J* = 8.9, 19.8	2α 2.72 *dd*, *J* = 9.7, 18.6
	2β 1.93 *dd*, *J* = 11.1, 19.8	2β 1.93 *dd*, *J* = 11.2, 19.8	2β 2.02 *dd*, *J* = 9.9, 18.6
4	3.05 *dq*, *J* = 1.8, 6.7	2.83 *dq*, *J* = 2.0, 6.6	3.13 *dq*, *J* = 2.1, 6.9
7	7α 2.54 *d*, *J* = 14.8	7α 2.57 *d*, *J* = 15.7	5.64 *d*, *J* = 1.9
	7β 1.55 *d*, *J* = 14.8	7β 1.50 *d*, *J* = 15.7	
10	5.74 *d*, *J* = 1.8	5.91 *d*, *J* = 2.0	5.85 *dq*, *J* = 1.9, 2.1
12	0.72 *s*	0.71 *s*	0.87 *s*
13	1.06 *d*, *J* = 6.7	1.05 *d*, *J* = 6.6	1.12 *d*, *J* = 6.4
14	0.75 *s*	0.76 *s*	0.99 *s*
15	1.08 *d*, *J* = 6.7	1.10 *d*, *J* = 6.6	1.20 *d*, *J* = 6.9
OMe		3.18 *s*	

* ^{1}H connectivities were determined by means of ^{1}H–^{1}H COSY and homodecoupling. The usual pulse sequences were used in NOESY.

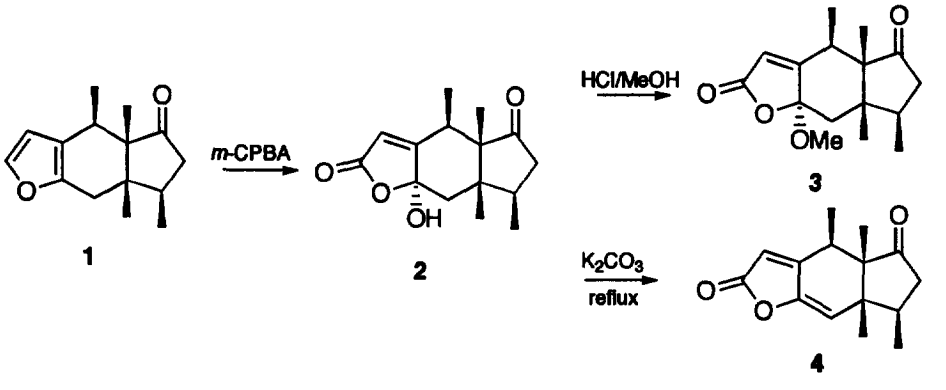
Table 2. ^{13}C NMR data for pinguisone derivatives (**2**–**4**)*

C	2	3	4
1	31.0	30.7	31.5
2	42.1	41.6	43.1
3	218.1	217.2	216.3
4	32.5	32.2	37.2
5	171.8	169.3	148.6
6	105.0	106.7	157.0
7	42.1	41.0	113.6
8	46.1	45.6	47.1
9	60.8	60.2	60.2
10	115.7	117.6	112.6
11	171.2	169.1	169.6
12	19.3	15.3	18.0
13	14.1	13.8	14.5
14	9.3	8.9	10.0
15	11.1	10.6	11.4
OMe		50.5	

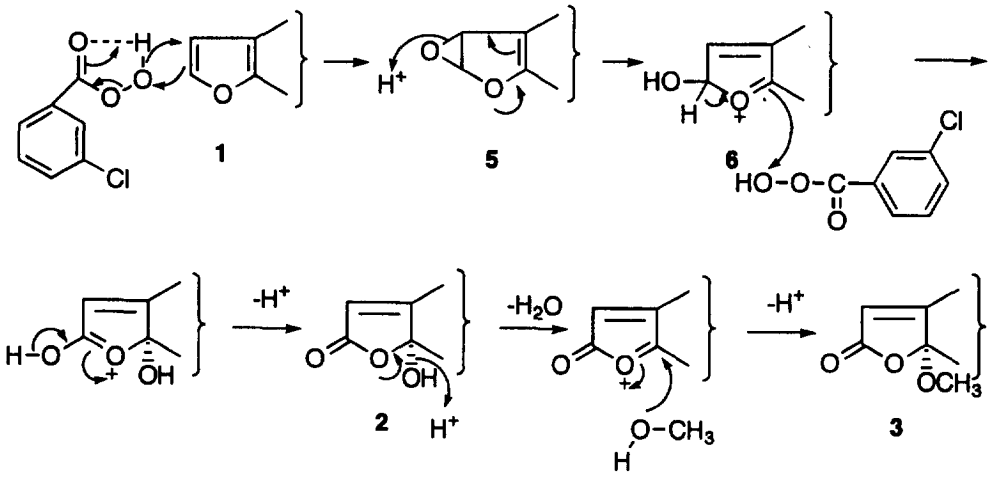
*67.8 MHz in $CDCl_3$; solvent peaks as the int. standard. Bond types were distinguished by DEPT. One-bond and long-range heteronuclear ^{1}H–^{13}C connectivities were determined by ^{1}H–^{13}C COSY and COLOC, respectively.

(H-12 to C-8; H-14 to C-1, C-8 and C-9; H-7 to C-6 and C-8) observed by COLOC. These facts indicated that **2** was an analogous compound to **1** where the furan ring had been replaced by a 6-hydroxy-11,6-γ-butenolide moiety. The long-range coupling between H-7α and H-10 resulted in a broad doublet of H-7α, showing that this hydrogen was equatorial. Proton–proton NOEs between H-7α and Me-12, between H-7α and Me-13, between H-7β and Me-12, and between Me-12 and Me-13 were observed by NOESY. From these observations, together with the high-field shift of H-7β (δ_H 1.55), the inspection of a model required the proposed configuration with the hydroxyl group at C-6 in the axial α-position. The oxidation of **1** with *m*-chloroperbenzoic acid (CPBA) gave further confirmation of the structural relationship between **1** and **2** (Scheme 1). This oxidation formed epoxide **5** at C-10/C-11 as an intermediate and was followed by further oxidation through oxomium cation **6** (Scheme 2). The stereoselectivity of the addition of the hydroxyl group to C-6 might be controlled by the steric hindrance of the axial H-7β proton.

Compound **3** was a colourless oil with a molecular formula, $C_{16}H_{22}O_4$ from the mass spectrum EI (*m*/*z* 279 $[M+1]^+$) and NMR data. The UV spectrum also showed an absorption at 235 nm (log ε 4.34) for an α,β-conjugated γ-lactone (1760 cm^{-1}). The comparison of all spectral data with those for **2** allowed the structure of **3** to be formulated as the methylated product of a hydroxyl group at C-6. Subsequent methylation of **2** with methanolic HCl afforded **3**, thus



Scheme 1. Chemical correlation of compounds **1**–**4**.



Scheme 2. Chemical conversion of pinguisone (1) via oxonium cations.

confirming the structural relationship between **2** and **3** (Schemes 1 and 2). The observed NOE between the methyl signal at δ_H 3.18 and H-4 signal at δ_H 2.83 indicated that the configuration at C-6 should be the same as that of **2**.

Compound **4** was a colourless oil with a molecular formula $C_{15}H_{18}O_3$ from the EI-HR mass spectrum (*m/z* 246.1233 $[M]^+$ calc. 246.1256). The UV spectrum showed an absorption at 278 (log ε 4.19) nm for an $\alpha,\beta,\gamma,\delta$-conjugated γ-lactone (1780 cm^{-1}). The ^1H and ^{13}C NMR, IR and mass spectra of **4** indicated that the 6-hydroxyl group of **2** was replaced by a C-6/C-7 double bond. The proton–proton long-range coupling between H-7 and H-10 strongly indicated the presence of a conjugated diene system in **4**, which is supported by the low-field shift of C-5 (δ_C 148.6) and C-6 (δ_C 157.0) signals. The structure for **4** was finally confirmed by the dehydration of **2** to **4** (Scheme 1). The absolute stereochemistry of **1** was established by an X-ray analysis of its *p*-bromobenzylidene derivative [15]. Thus, the absolute stereochemistry of **2**, **3** and **4** was established by the chemical correlation with **1**.

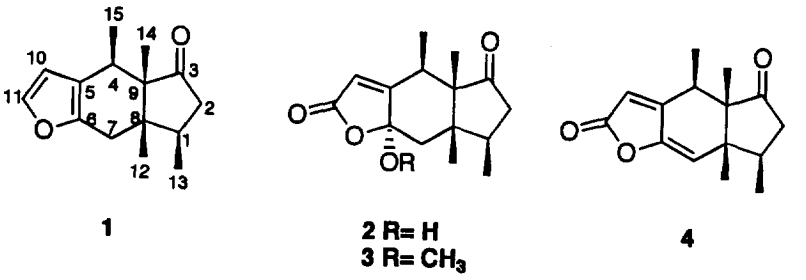
Dehydropinguisenol methyl ether, 6α,11α-dimethoxypinguis-5(10)-ene and 6α,11β-dimethoxypinguis-5(10)-ene have been isolated from *Trocholejeunea sandvicensis* as artefacts [10]. However, compounds **2**, **3** and **4** have been proved to be natural products by the following experiments: (i) they were detectable in diethyl ether extracts of freshly prepared plant materials by using GC; (ii) preparations of both **1** and **2** in methylene chloride–methanol solution with Sephadex LH-20 for three days generated no other modified compounds.

The structurally related sesquiterpene lactones, palmosalides and furodysisnins, have been isolated from the marine octocoral *Coelogorgia palmosa* [16] and the sponge *Hypselodoris zebra* [17], respectively. This was the first isolation of pinguisane-type γ-hydroxylactones from the liverworts, and the accomplishment of chemical conversion of **1** into its derivatives **3** and **4** is most probably like its biogenetic transformation in *A. pinguis*. In addition, *in vitro* cultures of bryophytes are useful for the production of plant material that otherwise has been available only in minor amounts from field collections [18].

EXPERIMENTAL

Optical rotations were measured in $CHCl_3$. UV spectra were measured in EtOH. NMR spectra were recorded in $CDCl_3$ soln using a 270-MHz instrument (H: 270 MHz; C: 67.5 MHz) relative to $CHCl_3$ at δ_H 7.26 and $CDCl_3$ at δ_C 77.0, respectively. ^{13}C multiplicities were determined using the DEPT pulse sequence. IR: KBr pellet.

The origin and axenic culture of *A. pinguis* have been previously reported [14]. Air-dried gametophytes (60 g) grown on B5 Gamborg medium were extracted with Et_2O in Soxhlet apparatus, then concd *in vacuo* to dryness (2.32 g). The Et_2O extracts were sepd into 5



fractions by CC on a Sephadex LH-20 column (25 mm i.d. × 150 cm) with CH_2Cl_2–MeOH (1:1) as eluent. The isolation of **1** (311 mg) from frs 3 and 4 has been previously reported [14]. Fr. 4 was further sepd by vacuum liquid chromatography on a silica gel column (20 mm i.d. × 10 cm) with *n*-hexane–EtOAc (stepwise) elution. Elution with *n*-hexane–EtOAc (4:1 and 7:3, 100 ml each) gave frs containing **3** and **4**. Elution with *n*-hexane–EtOAc (1:1, 100 ml) gave frs containing **2**. Frs containing **2** were further sepd by HPLC on silica gel (4.6 mm i.d. × 250 mm) with *n*-hexane–EtOAc (1:1) and afforded **2** (16.9 mg). Frs containing **3** and **4** were further sepd by HPLC with *n*-hexane–EtOAc (3:2) and afforded **3** (4.6 mg) and **4** (5.6 mg).

6α-Hydroxy-3-oxo-pinguis-5(10)-ene-11,6-olide (**2**). $[\alpha]_D$ −19.2° (*c* 0.24, $CHCl_3$); EIMS *m/z* (rel. int.): 264 (1), 246 (11), 202 (9), 190 (8), 176 (69), 161 (100), 148 (70), 133 (15), 121 (20), 105 (44), 98 (17), 91 (28), 77 (35), 69 (27), 53 (53), 41 (94); UV λ_{max} nm (log ε); 235 (4.48), 280 (3.67). IR ν_{max} cm^{-1}: 3350, 1730, 1650, 1450, 1380, 1230, 1160, 930. ^1H and ^{13}C NMR: see Tables 1 and 2, respectively.

6α-Methoxy-3-oxo-pinguis-5(10)-ene-11,6-olide (**3**). $[\alpha]_D$ −27.88° (*c* 0.165, $CHCl_3$); EIMS *m/z* (rel. int.): 279 (8), 263 (17), 195 (2), 167 (3), 151 (6), 137 (9), 124 (10), 109 (19), 95 (47), 81 (78), 67 (100), 55 (83), 41 (72). UV λ_{max} nm (log ε): 235 (4.34), 275 (3.76); IR ν_{max} cm^{-1}: 1760, 1730, 900. ^1H and ^{13}C NMR: see Tables 1 and 2, respectively.

3-Oxo-pinguis-5(10),6-diene-11,6-olide (**4**). $[\alpha]_D$ +106.0° (*c* 0.10, $CHCl_3$); EIMS *m/z* (rel. int.): 246 (64), 202 (18), 177 (36), 176 (100), 161 (70), 148 (46), 105 (13), 77 (7), 43 (8); UV λ_{max} nm (log ε); 278 (4.19), 214 (3.61); IR ν_{max} cm^{-1}: 1780, 1740, 1680, 1600, 1450, 1385, 1180, 890. ^1H and ^{13}C NMR: see Tables 1 and 2, respectively.

Oxidation of **1**. Compound **1** (4.1 mg) was oxidized with *m*-CPBA (14.4 mg) in CH_2Cl_2 at 30° for 1 hr. The reaction mixt. was washed with 5% $NaHCO_3$ and dried over Na_2SO_4. The resulting soln was evapd to dryness in vacuo and then subjected to CC on a silica gel column (3 mm × 3 cm), followed by HPLC (silica gel, 4.6 mm i.d. × 250 mm) with *n*-hexane–EtOAc (3:2) to afford purified **2** (3.0 mg).

Methylation of **2**. Compound **2** (3.3 mg) was dissolved in 1 ml 3% HCl–MeOH at room temp. After 1 hr, the reaction mixt. was diluted with 10 ml H_2O and then extracted (×3) with Et_2O (10 ml). The Et_2O extract was dried over Na_2SO_4 overnight and evapd *in vacuo* to dryness to afford purified **3** (3.3 mg).

Dehydroxylation of **2**. Compound **2** (2.6 mg) was dehydroxylated with K_2CO_3 (1.0 g) in Me_2CO by refluxing for 3 hr. The reaction mixt. was cooled to room temp. and filtered. The filtrate was evapd *in vacuo* to dryness, they resolved by CC on a silica gel column (3 mm × 3 cm), eluted with *n*-hexane–EtOAc (1:1), to afford purified **4** (1.8 mg).

GC analysis of terpenes. Cultured plant material (2–3 mg dry wt) was extracted with Et_2O. Tridecane was added as int. standard to quantify terpenes. The Et_2O extracts were evapd to small quantities and submitted to GC analysis. Analyt. GC was equipped with a FID and a cross-bonded 100% dimethyl polysiloxane Rtx-1 column (60 m × 0.25 mm i.d.). The column temp. was held at 60° for 5 min and elevated to 220° at 2° min^{-1}, with the flow rate of He being 1.20 ml min^{-1}. The peaks at 75.27, 87.47, 93.90 and 94.75 min were identified as **1**, **4**, **3** and **2**, respectively, by direct comparisons with authentic samples.

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